General and Comparative Endocrinology 176 (2012) 415–419

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

The decline in yolk progesterone concentrations during incubation is dependent on embryonic development in the European starling

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ARTICLE INFO

Article history:
Available online 22 December 2011

Keywords:
Yolk steroids
Maternal effects
Progesterone
Metabolism
Embryo
European starling

ABSTRACT

Oviparous amniotes, particularly birds, have become model systems in which to study how mothers may utilize steroids to adaptively adjust offspring development. Although there is now ample evidence that maternally derived steroids in the egg at oviposition can influence offspring phenotype, very little is known about how these steroids elicit such effects. Of the major avian steroid hormones found in yolk, progesterone is by far the most abundant at oviposition, but has received little research attention to date. In this study, we examine the metabolism of [^{3}H]-progesterone injected into freshly laid European starling eggs throughout the first 5 days of development by characterization of radioactivity within the egg homogenate. We also introduce a technique that utilizes a focal, freeze/thaw cycle to prevent embryonic development and allows us to assess the role of the embryo in metabolizing progesterone during early incubation. Two major findings result. First is that [^{3}H]-progesterone is metabolized in eggs possessing a developing embryo, but not in eggs with disrupted embryonic development. Second is that the change in the distribution of radioactivity within eggs possessing an embryo is the result of metabolism of [^{3}H]-progesterone to a more polar form that is subsequently conjugated. Together, these data suggest live embryos are necessary for metabolism of progesterone during early incubation, underscoring the potentially important contribution of embryos to functional modulation or mediation of maternal yolk steroid effects.

1. Introduction

The endocrine environment experienced by developing vertebrate embryos is vital to coordinating development. Variation in endocrine signals can create phenotypic variation that is either short-lived or persists throughout the lifetime of the organism, and many of the affected traits are thought to have fitness consequences making them potential targets of selection [9]. This is one reason researchers have taken an interest in how maternally derived steroids influence offspring phenotypes. Oviparous amniotes, particularly birds, have become model systems in which to study how mothers may utilize steroids to fine tune offspring development. That eggs, once laid, are independent of any direct maternal steroid signals facilitates experimental studies of this nature. And while there are now many examples of maternally derived steroids in the egg at oviposition influencing offspring development [6,8], very little is known about the mechanisms by which these steroids elicit such effects [4,9]. Understanding the mechanisms by which maternal steroids create phenotypic variation is critical to interpreting their adaptive potential [9].

Early studies of mechanism focused on how steroid concentrations in yolk change during development. The general trend is for concentrations to decline rapidly during the early part of development (reviewed in [16]). More recent investigations into the cause of this decline have shown that steroids are metabolized to various forms during the first several days of incubation. Both apolar steroids [18,21] as well as more polar steroid conjugates [15,18,21] have been identified as metabolites of exogenous steroids applied to eggs at the onset of incubation. In both the chicken (*Gallus gallus*) [21] and the European starling (*Sturnus vulgaris*) [18], there are more polar than apolar metabolites present in the egg after at least 5 days of incubation. In European starlings, testosterone is metabolized to a conjugated form of etiocholanolone *in ovo* over the first 6 days of incubation [18]. These data illustrate that both Phase I modification of steroids by enzymes such as 5β-reductase and Phase II metabolism by enzymes such as steroid sulfotransferases are occurring during early incubation.

Advancing our understanding of how steroid conjugation may modulate maternal steroid effects will facilitate interpretation of their potential physiological and evolutionary consequences. For instance, a conjugation pathway has recently been characterized in the developing embryos of a well-studied turtle where exogenous estradiol is primarily conjugated to estradiol sulfate [17]. Interestingly, administering exogenous estradiol sulfate was shown

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doi:10.1016/j.ygcen.2011.12.014

to influence offspring sex ratios demonstrating that at least some oviparous amniote embryos are either capable of converting steroid sulfates back to their parent form or are sensitive to the sulfates themselves [17].

To date, testosterone has been the most studied steroid present in yolk with respect to maternal effects in birds, but steroids such as estradiol, corticosterone, androstenedione, and 5α-dihydrotestosterone have also received some attention [9]. One steroid that has yet to receive much consideration is progesterone even though it is the most concentrated yolk steroid characterized to date in birds and reptiles (reviewed in [16]), and is found in highest concentration in the outermost layers of yolk [14,20]. Whole yolk progesterone levels in eggs of songbirds have been shown to increase across laying in a pattern similar to that commonly reported for yolk testosterone, but, again, at much higher concentrations [5]. The relationship between yolk progesterone and water content of the hatching muscle was examined in starlings, but no relationship was found to exist [13]. Another study in this species found that progesterone levels in the yolk decline, presumably due to metabolism, during embryonic development [18]. Yolk progesterone is an intriguing steroid to examine within the context of embryonic metabolism for several reasons. In addition to being the most concentrated yolk steroid, progesterone serves as a precursor in the production of other steroids such as testosterone, corticosterone, and estradiol (reviewed in [19]). Since both testosterone and progesterone can be metabolized by some of the same steroidogenic enzymes, the amount of progesterone in the yolk may influence testosterone metabolism due to enzyme kinetics or competition for cofactors involved in steroid metabolism. Metabolites of progesterone, such as pregnanediol and pregnanolone, have also been shown to produce biological effects such as increased porphyrin and hemoglobin synthesis in bird embryos [7,10,11]. Finally, progesterone metabolites (specifically 5β reduced metabolites) have been hypothesized to regulate steroid metabolism during embryonic development in amniotes by binding "xenobiotic-sensing nuclear receptors" such as the chicken xenobiotic receptor in birds [16]. Taken together, the existing data on yolk progesterone suggest that it is a steroid that warrants more attention.

In this study, we examine the metabolism of tritium-labeled progesterone injected into freshly laid European starling eggs during the first 5 days of development. We also describe a new technique that employs a repeated focal freeze/thaw cycle to prevent embryonic development and that allows us to test whether or not embryonic development is required for progesterone metabolism. We predict that eggs in which embryos do not develop will not metabolize progesterone while those with developing embryos and associated extraembryonic membranes will produce increased levels of metabolites along with a corresponding decrease in progesterone as development progresses. Characterizing the role of the embryo and its associated membranes in metabolism of yolk progesterone will help us determine the different pathways through which this steroid may influence offspring development. Moreover, determining whether or not embryos are actively involved in steroid metabolism and whether such embryonic involvement varies with individual circumstances may have important implications for elucidating the evolutionary consequences of maternal steroid transfer to yolk.

2. Materials and methods

Nine clutches of eggs were collected from free-living starlings nesting in boxes at the Illinois State University farm in Normal, IL. Eggs were collected between 21 April 2009 and 16 June 2009 by monitoring nest boxes daily and collecting freshly laid eggs and replacing them with painted wooden decoys that approximate the size, shape, and color of an average starling egg. Collected eggs were uniquely marked and then weighed within an hour of collection. All research was conducted according to all applicable federal, state, and university regulations. Egg collection procedures were approved by the Illinois State University Institutional Animal Care and Use Committee (protocol # 08-2008).

Entire clutches were assigned to one of two treatments. The first treatment contained five clutches of eggs and was designed to test for the presence of maternally derived enzymes that are capable of metabolizing progesterone without contribution from developing embryos or their associated extraembryonic membranes. Eggs were subjected to a focal freeze/thaw cycle meant to prevent embryonic and associated membrane development. Briefly, eggs were allowed to rest on their side with the long axis of the egg in the horizontal plane for a minimum of 60 s to allow the yolk to float to the top of the egg (Fig. 1). Eggs were then placed over a fiber optic light source while maintaining this horizontal orientation to allow visualization of the yolk within the egg. To freeze the embryo, dry ice wrapped in aluminum foil was applied to the top of the egg for approximately 30 s which resulted in the freezing of the top portion of the yolk. This frozen portion was allowed to thaw after which dry ice was reapplied to the egg for another 30 s. Ultimately, eggs were subjected to a total of three freeze/thaw cycles. After the final thaw, a 5 μl bolus of sesame oil containing 125,000 cpm of [^{3}H]-progesterone was injected into the albumen and allowed to float to the top of the egg where it came into close contact with the top of the yolk [18,21]. The second treatment contained four clutches of eggs that were similarly manipulated by placing them in the horizontal orientation described above but they were not subjected to any freeze/thaw cycle to allow normal embryonic development to proceed. These eggs were also injected with 125,000 cpm of [^{3}H]-progesterone as described above. Eggs from both treatments were then incubated at 37.5 °C with 60% humidity (Turn-X Model TX7 incubator, Lyon Technologies Inc., Chula Vista, CA, USA).

One egg from each clutch was randomly assigned to be sampled on one of the first 5 days of development. On the appropriate sampling day, eggs were removed from the incubator and placed at −20 °C for storage until metabolite identification. To characterize any metabolism of progesterone that may have occurred over the first 5 days of development, eggs were removed from −20 °C and thawed. Upon thawing, eggs were inspected for the presence of an embryo, associated extraembryonic membranes, and vascularization of yolk. The entire content of the egg (excluding the shell)

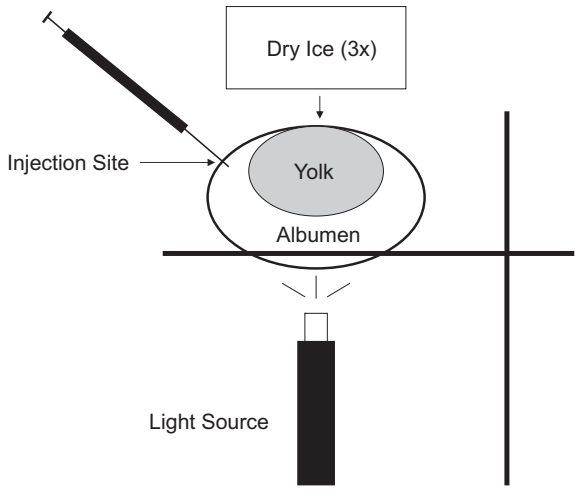

Fig. 1. Diagram of the techniques used to prevent embryonic development and to inject [^{3}H]-progesterone.

was then homogenized using a Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK, USA). The resulting homogenate included yolk, albumen, and any embryonic tissue that may have been present. From each homogenate, a 100 mg aliquot was subjected to an ether extraction to distinguish levels of apolar (free) steroids and polar (conjugated) steroids [18,21]. Aliquots were added to 1 ml of distilled water, vortexed, and allowed to equilibrate at 4 °C overnight. Apolar steroids were then extracted twice with 3 ml of ether extraction solvent (30% petroleum ether and 70% diethyl ether). The ether fraction was dried under nitrogen gas and reconstituted in 1 ml of 90% ethanol. Radioactivity levels were then counted in two 200 μl aliquots of both the ether fraction and the aqueous fraction in a scintillation counter. Total radioactivity of each homogenate was calculated by multiplying the mean cpm in 100 mg by the total homogenate mass.

Radioactive metabolites in the eggs that were incubated 5 days were further characterized. These eggs were selected for further analysis based on the assumption that a longer incubation period would lead to more metabolism of the injected [^{3}H]-progesterone. Here, five 100 mg aliquots of each Day 5 egg homogenate were subjected to the ether extraction described above. The ether fractions were then dried under nitrogen gas while the aqueous fractions were subjected to acid solvolysis to return conjugated steroids in this fraction back to their parent (free) form. Solvolysis was conducted by drying the aqueous fraction under nitrogen gas and reconstituting the residue in 200 μl of 2.5 M H_2SO_4 and 400 μl of saturated NaCl solution and incubating a 37 °C overnight [3,18]. The newly freed steroids were then extracted twice with 1.5 ml of ether extraction solvent. This ether fraction containing the previously conjugated steroids was dried under nitrogen gas. The five initially ether-soluble and five post-solvolysis ether-soluble fractions from each egg were combined in 1 ml of 10% ethyl acetate in isooctane to yield a single non-solvolysis and a single post-solvolysis sample for each egg homogenate. Steroids within these samples were then fractionated via Celite chromatography [1,18,22]. Four concentrations of ethyl acetate in isooctane were sequentially passed through the column and collected separately to isolate several steroids (progesterone and androstenedione = 2%, etiocholanolone = 10%, testosterone = 20%, and estradiol = 40%).

Radioactivity levels in each of the eluates from the Celite chromatography were determined by drying each eluate under nitrogen gas, reconstituting it in 1 ml of 90% ethanol, and counting the radioactivity in two 200 μl aliquot. Eluates containing substantial amounts of radioactivity were subjected to quantitative thin-layer chromatography (TLC) for further characterization. The remaining sample was dried and spotted onto aluminum-backed silica TLC plates and developed in cyclohexane:ethyl acetate (1:1) [18,21]. Standards of progesterone, testosterone, and androstenedione were also spotted along with the sample and could be visualized under UV light so their length of migration on the plate could be noted. To characterize the migration of radioactive progesterone and its metabolites on the TLC plate, a 1 cm wide lane that ran the entire length of the plate and contained the sample and standards was excised from the plate. This lane was then cut into 0.5 cm long sections that were added to 3.5 ml of scintillation fluid and counted. Co-migration of radioactivity with a standard was used as evidence of the identity of the radioactive moiety.

To test for differences in radioactivity levels between the two treatments across the first 5 days development, we used mixed-model ANOVAs to compare levels in the ether fraction and the aqueous fraction, respectively. Treatment, day of development, and their interaction were included as fixed factors. Clutch of origin was included as a random factor. Egg mass was initially included in the model as a covariate but was ultimately removed due to a lack of significance ($P = 0.33$). Radioactivity levels were log transformed prior to analysis and *post hoc* (Tukey's HSD) were used to compare differences between groups. All statistical tests were performed in SAS v. 9.1 (SAS Institute, Cary, NC, USA).

3. Results

Subjecting the eggs to freezing was successful in preventing embryonic development as 0% (0/11) of the eggs in this treatment group possessed an embryo, extraembryonic membranes, or vascularized yolk after three or more days of incubation. In the live treatment group, 92% (12/13) of the eggs that were incubated at least 3 days exhibited normal development characterized by development of embryos and associated membranes and a vascularized yolk sac. For eggs only incubated 1 or 2 days, there was not sufficient development to accurately determine whether or not a viable embryo was present, so, for assessment of steroid metabolism, all of the eggs from the live treatment were assumed to possess a developing embryo and all of the eggs from the freezing treatment were assumed to exhibit no embryonic development. This was a conservative approach, as some eggs with unviable embryos may have been included in the live embryo treatment, which would be expected to increase variability within that group and lessen our ability to detect a treatment difference. A single egg in the live treatment group that was incubated for 4 days but did not develop was omitted from the study.

The embryo treatment influenced radioactivity levels in both the ether and aqueous fraction across the first 5 days of development. In the ether fraction, treatment ($F_{1,21} = 33.6$, $P < 0.0001$), day of development ($F_{4,21} = 3.8$, $P = 0.018$), and their interaction ($F_{4,21} = 7.25$, $P = 0.0008$) significantly affected radioactivity levels. *Post hoc* comparisons indicate that the distribution of radioactivity between aqueous and organic compartments of the live eggs change during development while distribution in the dead eggs does not change (Table 1 and Fig. 2). In the aqueous fraction, day of development ($F_{4,21} = 31.1$, $P < 0.0001$) and its interaction with treatment ($F_{4,21} = 11.5$, $P < 0.0001$) had a significant effect on radioactivity levels while treatment ($F_{1,21} = 0.01$, $P = 0.98$) did not have an effect. As in the organic fraction, *post hoc* comparisons indicate that the distribution of radioactivity in the live eggs changes during development while it does not in eggs with dead embryos (Table 1).

Characterization of the metabolites in the eggs incubated 5 days suggested that the radioactivity in the initial ether fraction was predominantly [^{3}H]-progesterone. The radioactivity in this fraction (from both treatments) eluted in the 2% ethyl acetate in isooctane fraction within which progesterone and androstenedione are known to elute (Table 2). The radioactivity in this fraction also co-migrated with the progesterone standard and not the androstenedione standard on the TLC plates. Very little radioactivity was detected in any of the other eluates from the Celite chromatography (Table 2). However, radioactivity was present in the post-solvolysis ether extract from the live eggs prior to loading it onto the Celite columns. Follow-up tests determined that this radioactivity did not elute, but, instead, remained in the Celite column during elution.

4. Discussion

Two major findings resulted from this experiment. The first is that the distribution of radioactivity within eggs differed depending on whether or not embryonic development occurred. In eggs with developing embryos, a substantial amount of radioactivity shifted from being ether-soluble to being water-soluble, but this shift did not occur in eggs that were not developing. The second is that the change in solubility of radioactivity within eggs containing live embryos and developing extraembryonic membranes was

Table 1
Total radioactivity levels (mean (SE)) within whole egg homogenates over the first 5 days of incubation. Significant differences between days of development are indicated for live eggs (capital letters) and dead eggs (lower-case letters). Within each treatment, days not sharing a letter are significantly different ($P < 0.05$).

Incubation day	Alive treatment			Dead treatment		
	N	Ether-soluble	Water-soluble	*N*	Ether-soluble	Water-soluble
1	5	79,434 (5952)^{a}	12,976 (546)^{A}	3	99,714 (7553)^{a}	21,437 (2699)^{A}
2	5	83,113 (1418)^{a}	16,150 (1326)^{A}	2	75,096 (8567)^{a}	24,782 (3250)^{A}
3	5	67,849 (4301)^{a}	22,134 (1334)^{B}	3	77,246 (3197)^{a}	19,014 (859)^{A}
4	3	56,230 (2131)^{a,b}	32,656 (5470)^{C}	4	90,681 (14,789)^{a}	22,825 (2333)^{A}
5	4	46,747 (3037)^{b}	38,793 (5131)^{C}	4	95,849 (10,329)^{a}	28,198 (3519)^{A}

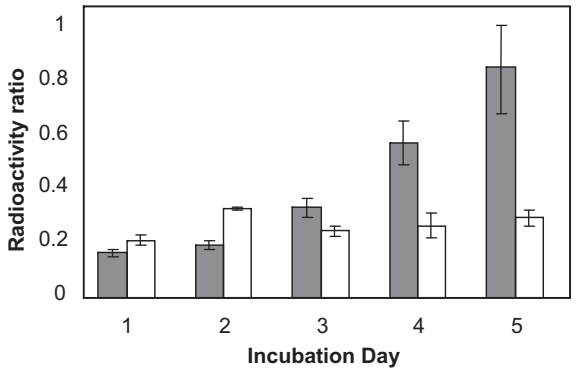

Fig. 2. Graph depicting the ratio of water-soluble radioactivity to ether-soluble radioactivity within the whole egg homogenates from eggs with (gray bars) and without (open bars) developing embryos.

Table 2
Mean radioactivity (cpm ± SE) levels from each of the eluates resulting from Celite chromatography of Day 5 egg homogenates.

		Eluate			
		2	10	20	40
Non-solvolysis	Dead	2983 (333)	94 (11)	85 (11)	70 (9)
Non-solvolysis	Live	1534 (74)	164 (12)	63 (2)	39 (2)
Post-solvolysis	Dead	237 (35)	24 (3)	18 (1)	18 (2)
Post-solvolysis	Live	102 (3)	38 (2)	33 (2)	28 (1)

the result of [^{3}H]-progesterone being metabolized to a more polar form that was subsequently conjugated. This was demonstrated first by the return of most aqueous radioactivity back to an ether-soluble (free) form following acid solvolysis, and then by ether-soluble radiation failing to elute from the Celite column during chromatography, presumably due to high polarity. We did not determine the specific identity of the radioactive metabolite(s) left on the column, but did confirm that the radioactive moiety was no longer progesterone.

Previous work has shown that the total yolk progesterone declines in starling eggs as development proceeds [18]. Data from this experiment show that live eggs are capable of metabolizing progesterone and that the observed decline in detectable progesterone is dependent on factors of embryonic origin, and does not occur in the absence of embryonic development. Demonstrating that eggs lacking both developing embryos and their associated extraembryonic membranes do not metabolize progesterone is relevant to interpreting recent studies that have shown steroid metabolism takes place in bird [18,21] and turtle [15,17] eggs during the early part of development. The experimental design of these previous studies could not rule out the possibility that this metabolism was due solely to maternal contributions to eggs. Our data suggest that the embryo or its extraembryonic membranes are required for this early steroid metabolism, at least for progesterone.

In addition to providing data on progesterone metabolism by avian embryos, we also describe a novel technique that can be used to prevent embryonic development in eggs. Subjecting the top of the yolk to repeated freeze/thaw cycles was 100% effective in terminating embryonic development, and focal freezing did not lead to any obvious disruption of the vitelline membrane that occurs during freezing of the entire egg (personal observation). This minimally invasive technique can be used by researchers wishing to test the effects of incubation on eggs that do not possess a developing embryo. By utilizing this technique, we were able to demonstrate that embryonic development is required for the *in ovo* metabolism of progesterone in European starlings.

Evidence that embryos of oviparous amniotes metabolize maternal steroids continues to mount [15,17,18,21]. One similarity in all of these studies is that steroids are converted to more polar moieties during the early part of development. While it was not confirmed in most studies, steroid conjugation was thought to be the mechanism behind this conversion. From what we currently know about how oviparous amniote embryos metabolize maternal steroids, it appears that a similar mechanism to that found in placental mammals is employed. In placental species, steroid conjugation (primarily sulfonation) is known to be critical to regulating steroids as they pass from mother through the placenta to the offspring, and vice versa [12]. Importantly, the resulting steroid sulfates can be returned to their active form by steroid sulfatase (EC 3.1.6.2) resulting in a dynamic activation/deactivation interplay between mother and offspring. While a similar interplay is not possible in oviparous amniotes, it appears that embryonic processes such as steroid metabolism also may play a role in the modulation of steroid-mediated maternal effects.

At this point, more work is needed to understand the role of embryonic steroid metabolism and where within the egg it occurs. Cultured extraembryonic membranes from chicken eggs exhibit Phase I steroid metabolism of steroid precursors to progesterone during middle to late stages of embryonic development [2], a finding which suggests that a good deal of steroid metabolism in eggs may occur outside the developing embryo. One critical question that remains unanswered is whether or not these steroid metabolites influence embryonic development. In starlings, steroids appear to be metabolized prior to conjugation as the major metabolite of testosterone was a conjugated form of etiocholanolone [18] and the major metabolite of progesterone in this study was a conjugated form(s) of steroid more polar than progesterone. Steroid metabolites such as etiocholanolone and the unidentified progesterone metabolite(s) from this study may exert an effect prior to conjugation, the conjugate itself may exert an effect, the conjugate may be reactivated to exert an effect, or the metabolites may be inactive and exert no effects at all. With the exception of starlings [18] and chickens [21], very little is known about *in ovo* steroid metabolism in birds. Data from other species are needed to determine the generality of metabolic pathways *in ovo*. Finally, even though data from this study demonstrate that the metabolism of progesterone requires a developing embryo, maternal influences on this metabolism may still be possible. Metabolism may vary

depending on factors such as yolk steroid levels or incubation temperature. Future studies of *in ovo* yolk steroid metabolism should continue to advance our understanding of the potential evolutionary consequences of steroid-mediated maternal effects.

Acknowledgments

The authors are grateful to Rachel Bowden for the use of space and equipment and to Ellen Ketterson for the use of the incubator. The authors also thank the staff at the Illinois State University farm for logistical support. Financial support for this research was provided by the Illinois State University School of Biological Sciences to J.M.C. R.T.P. is currently supported by National Science Foundation Grant IOS-0952840.

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